

FORM PTO-1390 (7-97) (Rev. 10/95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

RDID0011US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/485896

INTERNATIONAL APPLICATION NO.

PCT/EP98/05242

INTERNATIONAL FILING DATE

18 August 1998

PRIORITY DATE CLAIMED

19 August 1997

TITLE OF INVENTION

PROCESS FOR THE ENZYMATIC PRODUCTION OF GUANOSINE DIPHOSPHATE-6-DEOXYHEXOSES AND
THEIR USE FOR THE PRODUCTION OF OLIGOSACCHARIDES

APPLICANT(S) FOR DO/EO/US

Roche Diagnostics GmbH

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). (unexecuted).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Statement to Support Filing and Submission in Accordance with 37 C.F.R. Sections 1.821-1.825 (2pp) in duplicate,
Sequence Listing (4pp) and computer readable diskette;
Notification of the Recording of a Change mailed 4 February 1999 (1pp);
Notification of the Recording of a Change mailed 17 August 1999 (1pp);
General Appointment of Representative for U.S. Patent and Patent Application (1pp); and
Return postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

09/7485896

PCT/EP98/05242

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20. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

\$840.00

- ☒ Search Report has been prepared by the EPO or JPO ~~\$930.00~~
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ~~\$670.00~~
~~\$720.00~~
- ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ~~\$690.00~~
~~\$790.00~~
- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ~~\$970.00~~
~~\$1,070.00~~
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ~~\$96.00~~
~~\$98.00~~

CALCULATIONS PTO USE ONLY**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$840.00

Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).

☐ 20 ☐ 30

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	26 - 20 =	6	x \$18.00
Independent claims	7 - 3 =	4	x \$78.00

\$108.00

\$312.00

Multiple Dependent Claims (check if applicable) ☐

\$0.00

TOTAL OF ABOVE CALCULATIONS =

\$1,260.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). ☐

\$0.00

SUBTOTAL =

\$1,260.00

Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).

☐ 20 ☐ 30

\$0.00

TOTAL NATIONAL FEE =

\$1,260.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐

\$0.00

TOTAL FEES ENCLOSED =

\$1,260.00

Amount to be:	\$
refunded	
charged	\$

A check in the amount of _____ to cover the above fees is enclosed.

- ☒ Please charge my Deposit Account No. **50-0877** in the amount of _____ to cover the above fees.
 A duplicate copy of this sheet is enclosed.

- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0877**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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30,444

REGISTRATION NUMBER

17 February 2000

DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Wolfgang Piepersberg, *et al.*

Application No.: To Be Assigned

Group No.: To Be Assigned

Filed: February 17, 2000

Examiner: To Be Assigned

For: Process For The Enzymatic Production Of Guanosine Diphosphate-6-Deoxyhexoses And Their Use For The Production Of Oligosaccharides

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please enter the following amendments prior to examination of the above-referenced application:

IN THE CLAIMS:

Please cancel claims 1 to 13 in the translated application filed herewith.

Please add the following new claims:

14. A process for the production of a guanosine diphosphate(GDP)-6-deoxyhexose comprising:
 - a. incubating a starting substance selected from the group consisting of GDP-D-mannose, precursors and secondary products thereof with an enzyme having activity selected from the group consisting of phosphomannomutase (manB), GDP-D-mannose synthase (manC), GDP-D-mannose-4,6-dehydratase (gmd, rfbD), GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (wcaG)

and GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase (rfbE) activity, and

b. isolating said GDP-6-deoxyhexose.

15. The process of claim 14, wherein said GDP-6-deoxyhexose is selected from the group consisting of GDP-4-keto-6-deoxy-D-mannose, GDP-L-fucose and GDP-D-perosamine and wherein said starting substance is GDP-D-mannose.
16. The process of claim 14, wherein said enzyme has GDP-D-mannose-4,6-dehydratase (gmd, rfbD) activity and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (wcaG) activity.
17. The process of claim 14, wherein said enzyme has GDP-D-mannose-4,6-dehydratase (gmd, rfbD) activity and GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase (rfbE) activity.
18. The process of claim 14, wherein said enzyme is obtained by cloning a gene coding for said enzyme, inserting said gene into a vector, transforming said vector into a host cell, overexpressing said enzyme and isolating said enzyme.
19. The process of claim 18, wherein said gene is amplified prior to said cloning.
20. The process of claim 18, wherein said gene is selected from the group consisting of *manB*, *manC*, *gmd*, *rfbD*, *rfbE* and *wcaG*.
21. The process of claim 18, wherein said host cell is selected from the group consisting of *E. coli*, *Bacillus subtilis*, *Corynebacterium sp.*, *Staphylococcus carnosus*, *Streptomyces lividans*, *Saccharomyces cerevisiae*, *Schizosaccaramyces pombe*, *Hansenula polymorpha*, and *Pichia stipidis*.
22. The process of claim 14, wherein said GDP-6-deoxyhexose is selected from the group consisting of GDP-D-mannose and secondary products thereof, said starting substance comprises D-mannose-6-phosphate and guanosine triphosphate (GTP), and said enzyme comprises phosphomannomutase (manB) and GDP-D-mannose

synthase (manC).

23. The process of claim 18, wherein said process is carried out as a batch process.
24. The process of claim 18, wherein said process is carried out continuously in an enzyme-membrane reactor.
25. The process of claim 18, wherein said enzyme is immobilized on a solid support and wherein a buffer solution comprising said starting substance is continuously percolated thereover.
26. A process for the in vivo production of a fucosylated end product comprising:
 - a. cloning a gene coding for a protein having wcaG activity, transforming said gene into a host cell and overexpressing said wcaG in said host cell,
 - b. incubating intracellularly the wcaG produced in step a with GDP-4-keto-6-deoxy-D-mannose and NADPH₂ to produce GDP-L-fucose, and
 - c. transferring intracellularly the GDP-L-fucose produced in step b onto a substrate selected from the group consisting of glucosides, oligosaccharides and polysaccharides with the aid of a fucosyltransferase to form said fucosylated end product.
27. A process for the in vivo production of a perosaminylated end product comprising:
 - a. cloning a gene coding for a protein having rfbE activity, transforming said gene into a host cell and overexpressing said rfbE in said host cell,
 - b. incubating intracellularly the rfbE produced in step a with GDP-4-keto-6-deoxy-D-mannose and L-glutamate to produce GDP-D-perosamine, and
 - c. transferring intracellularly the GDP-D-perosamine produced in step b onto a substrate selected from the group consisting of glucosides, oligosaccharides and polysaccharides with the aid of a perosaminyltransferase to form said

perosaminylated end product.

28. The process of claim 26, wherein said protein having activity is derived from an organism selected from the group consisting of *Yersinia enterocolitica* and *Escherichia coli*.
29. The process of claim 27, wherein said protein having activity is derived from *Vibrio cholerae* 01.
30. The process of claim 26 or 27, wherein said host cell is selected from the group consisting of *Escherichia coli*, *Streptomyces sp.* and *Saccharomyces cerevisiae*.
31. A process for the production of GDP-D-mannose comprising:
 - a. incubating D-mannose-6-phosphate and GTP with enzymes having manB and manC activity, wherein said enzymes are produced by cloning genes coding for manB and manC, transforming said genes into a host cell, overexpression of manB and manC in said host cell and isolation of said enzymes, and
 - b. isolating said GDP-D-mannose.
32. A process for the production of GDP-4-keto-6-deoxy-D-mannose comprising:
 - a. incubating GDP-D-mannose with an enzyme having activity selected from the group consisting of gmd and rfbD, wherein said enzyme is produced by cloning a gene coding for said activity, transforming said gene into a host cell, overexpression of said activity in said host cell and isolation of said enzyme, and
 - b. isolating said GDP-4-keto-6-deoxy-D-mannose.
33. A process for the production of GDP-L-fucose comprising:
 - a. incubating GDP-4-keto-6-deoxy-D-mannose and NADPH₂ with an enzyme having wcaG activity, wherein said enzyme is produced by cloning a gene

coding for said wcaG, transforming said gene into a host cell, overexpression of said wcaG in said host cell and isolation of said enzyme, and

b. isolating said GDP-L-fucose.

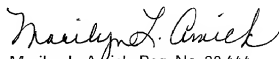
34. A process for the production of GDP-D-perosamine comprising:

a. incubating GDP-4-keto-6-deoxy-D-mannose and L-glutamate with an enzyme having rfbE activity, wherein said enzyme is produced by cloning a gene coding for said rfbE, transforming said gene into a host cell, overexpression of said rfbE in said host cell and isolation of said enzyme, and

b. isolating said GDP-D-perosamine.

35. The process of any of claims 31-34, wherein said enzyme is immobilized on a solid support.

Respectfully submitted,



Date: February 17, 2000

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Assistant Commissioner for Patents
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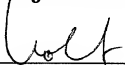
**GENERAL APPOINTMENT OF REPRESENTATIVE FOR
U.S. PATENT AND PATENT APPLICATION**

The undersigned applicant or assignee hereby appoints D. Michael Young, Reg. No. 33,819, Richard T. Knauer, Reg. No. 35,575, Brent A. Harris, Reg. No. 39,215, Jill Lynn Woodburn, Reg. No. 39,874, and Marilyn L. Amick, Reg. No. 30,444 all of Roche Diagnostics Corporation, 9115 Hague Road, P.O. Box 50457, Indianapolis, Indiana 46250, Telephone (317) 845-2000:

to prosecute and transact all business on its behalf before the United States Patent and Trademark Office in connection with any U.S. patent assigned to it and any U.S. patent application filed by it or on its behalf and to receive payments on its behalf.

Signed this 24th day of November, 1999 at Mannheim, Germany.

Roche Diagnostics GmbH



Signature

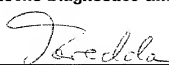
Dr. Bernd Kolb

Print Name

Senior Vice President

Position or Title

Roche Diagnostics GmbH



Signature

Dr. Andreas Poreda

Print Name

Director

Position or Title

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Process for the enzymatic production of guanosine diphosphate-6-deoxyhexoses and their use for the production of oligosaccharides

The present invention concerns a process for the enzymatic synthesis of guanosine-diphosphate (GDP)-6-deoxyhexoses, for example GDP-4-keto-6-deoxy-D-mannose, GDP-L-fucose and GDP-L-perosamine, from simple nutrients or from GDP-D-mannose. The process also concerns the use of GDP-6-deoxyhexoses formed in microorganisms or in vitro for the synthesis of oligosaccharides or polysaccharides using glycosyltransferases.

Hexoses which occur in large quantities in nature such as D-glucose, D-glucosamine, D-mannose and D-galactose are incorporated into oligosaccharides or polysaccharides by firstly activating them by means of nucleoside triphosphates (NTP) to form NDP-hexoses (UDP-, dTDP-, GDP-, CDP-hexoses) and subsequently transferring them to corresponding precursor molecules by specific glycosyltransferases. However, these simple sugars are often firstly biosynthetically converted into secondary modified NDP-activated hexose derivatives. A large group of these sugar molecules, NDP-6-deoxyhexoses (usually dTDP-, GDP- or CDP-activated hexose derivatives), is characterized by the loss of the 6-hydroxyl group and is incorporated in various modifications into many biological molecules with a characteristic function. Typical representatives of this group of substances are L-fucose (from GDP-D-mannose), L-rhamnose (from dTDP-D-glucose) and 3,6-dideoxyhexoses of the enterobacteria

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(e.g. D-colitose from CDP-D-glucose). Sugar derivatives produced from GDP-D-mannose such as L-fucose and D-perosamine are produced by means of numerous enzymes (ca. 30), which are often end-product-inhibited. In this connection those enzymes are advantageous for over-production in a host organism which exhibit only a weak or practically no feedback inhibition such as ManB (phosphomannomutase), ManC (mannose-1-phosphate guanylyltransferase [GDP-mannose pyrophosphorylase or synthase]) and enzymes from the gram-negative bacterium *Escherichia coli* (Stevenson, G. et al., J. Bacteriol. 178 (16), 4885-4893, 1996).

The biosynthetic pathway of the 6-deoxyhexoses typically starts with a dehydration reaction catalysed by an NAD(+)-dependent NDP-hexose-4,6-dehydratase to form an NDP-6-deoxy-D-4-hexulose (Figure 1; Piepersberg, W., Crit. Rev. Biotechnol. 14, 251-285, 1994; Liu, H.-W. and Thorson, J.S., Ann. Rev. Microbiol. 48, 223-256, 1994; Piepersberg, W. and Distler, J. In: Biotechnology (2nd Ed.), Vol. 7, Products of Secondary Metabolism (Rehm, H.-J., Reed, G., Pühler, A., Stadler, P., Eds.), p. 397-488, 1997). Further modification reactions can occur at the level of the D-configuration and use for example the 4-keto compound either for transamination, dehydroxylation of the neighbouring 3-hydroxy group by a dehydrase reaction or for enantioselective reduction to form the 4-hydroxy group. Other biosynthetic pathways firstly convert NDP-6-deoxy-D-4-hexulose by means of an epimerase reaction into an NDP-6-deoxy-L-4-hexulose in the process of which the stereospecificity of position 3 of the hexose is usually reversed in addition to that of position 5 (figure 1).

The direct production of fucose from mannose can only

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occur in metabolism in the nucleotide-activated form. The biosynthetic enzymes therefore depend on an activation i.e. the nucleotide moiety. Other secondary i.e. strongly modified sugars are also biosynthesized in the nucleotide-activated form as shown for example for dTDP-L-rhamnose (DE 195 37 217.4; Verseck, S., Dissertation, University, Wuppertal, 1997). In this connection dTDP-6-deoxy-D-xylo-4-hexulose is an intermediate product of the biosynthetic pathway. The enzymatic synthesis and isolation of this substance has also been described (Marumo, K. et al., Eur. J. Biochem. 204, 539 - 545, 1992).

GDP-L-fucose is for example produced from GDP-D-mannose in two to three successive enzymatic steps as for example shown by Chang et al. using pig salivary glands as an example (J. Biol. Chem., 263, 1693-1697, 1988) and which has been assumed in analogy to the biosynthesis of L-rhamnose and L-(dihydro)streptose in bacteria (Marumo, K. et al., Eur. J. Biochem. 204, 539 - 545, 1992; Verseck, S., Dissertation, Wuppertal, 1997). On the basis of genetic evidence in *Escherichia coli* Reeves et al. (J. Bacteriol. 178, 4885-4893, 1996) assume that the two genes *gmd* (GDP-D-mannose-4,6-dehydratase) and *wcaG* (GDP-4-keto-6-deoxy-D-mannose 3,5-epimeraseII-4-keto reductase) in the gene cluster for the synthesis of cholanolic acid are sufficient for the conversion into GDP-L-fucose. Consequently, in contrast to the synthesis of dTDP-L-rhamnose from dTDP-D-glucose, two instead of three enzymes are sufficient for the synthesis of GDP-L-fucose from GDP-D-mannose (Marumo, K. et al., Eur. J. Biochem. 5, 204, 539-545, 1992; Verseck, S., Dissertation, Wuppertal, 1997). Moreover Chang et al. (J. Biol. Chem. 263, 1693-1697, 1988) have already described the conversion of GDP-D-mannose by means of a

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specific dehydratase into firstly GDP-4-keto-6-deoxy-D-mannose which is subsequently converted by means of an enzyme having epimerase and ketoreductase activity into GDP-L-fucose. Furthermore other deoxyhexoses are probably derived from the common intermediate product GDP-4-keto-6-deoxy-D-mannose such as the transamination product GDP-D-perosamine which is attributed to the presence of the gene *rfbE* in the enterobacterium *Vibrio cholerae* (Manning, P.A. et al., Gene 158, 1-7, 1995). In contrast the direct production of L-fucose, D-perosamine and other derivatives of the GDP-6-deoxyhexose-biosynthetic pathway starting with D-mannose is not possible in quantities of economic interest in the absence of the specific nucleotide group.

L-fucose and D-perosamine are important building blocks of extracellular polysaccharides, glycoproteins and other cell surface glycoconjugates such as tetra-saccharides, like sialyl LewisX. Furthermore L-fucose and D-perosamine are important components of other natural substances such as the macrolide antibiotic perimycin. The transfer of sugars from the GDP-activated precursors into such (pseudo)saccharidic end products is usually carried out by specific glycosyl-transferases. Diverse sources for fucosyltransferases which include human samples are known: Fut1 (Flegel W.A., Dissertation University Ulm, 1998), Fut2 (Kelly, R.J. et al., J.Biol. Chem. 270 (9), 4640-4649, 1995) or Fut3 (Cameron, H.S. et al., J. Biol. Chem. 270 (34), 20112-20122, 1995) and various bacteria e.g. from *Escherichia coli* (Stevenson, G. et al., J. Bacteriol. 178 (16), 4885-4893 (1996), from *Yersinia enterocolitica* (Zhang, L. et al., Mol. Microbiol. 23, 63-76, 1997) or from *Helicobacter pylori* (Martin, S.L., Glycobiology (in print)).

Hence the object of the invention was to provide a simple process with the least possible number of steps for the production of guanosine diphosphate (GDP)-6-deoxyhexose compounds.

The object is achieved by a process for the enzymatic production of a GDP-D-hexose in which GDP-D-mannose or a compound that can be converted into GDP-D-mannose is incubated as the starting compound in the presence of an enzyme or several enzymes that have GDP-D-mannose-4,6-dehydratase (Gmd, RfbD) and optionally GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (WcaG) or GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase (RfbE) activity and the desired product is isolated. The enzymes required for the enzymatic synthesis are preferably isolated according to the invention by cloning the genes or DNA fragments coding for these enzymes, insertion into one or several vector(s) and transformation into a bacterial or fungal host cell. The process is especially suitable for the preparative in vitro production and purification of GDP-4-keto-6-deoxy-D-mannose or GDP-L-fucose by overproduction of the corresponding biosynthetic enzymes in suitable host organisms such as in *E. coli*. The biosynthetic enzymes are phosphomannomutase (ManB), GDP-D-mannose synthesis (ManC or pyrophosphorylase, mannose-1-phosphate guanyltransferase), GDP-D-mannose-4,6-dehydratase (Gmd, RfbD) and/or GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-ketoreductase (WcaG, or GDP-L-fucose synthase) which are preferably derived from *E. coli* (Stevenson, G. et al., *J. Bacteriol.* 178, 4885-4893, 1996).

In addition the process according to the invention is suitable for the preparative production of GDP-D-perosamine in which there is an overproduction of GDP-D-

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perosamine synthase (RfbE, GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase) from *Vibrio cholerae* O1.

Consequently the following genes or DNA fragments coding for the corresponding enzymes are preferred according to the invention: *manB*, *manC*, *gmd*, *rfbD*, *rfbE* and *wcaG*. If necessary the genes or appropriate DNA regions are specifically amplified, e.g. by suitable primers, in a PCR reaction before they are used for expression.

Suitable bacterial or fungal host organisms are for example *E. coli*, *Bacillus subtilis*, *Corynebacterium* sp., *Staphylococcus carnosus*, *Streptomyces lividans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Hansenula polymorpha* and *Pichia stipidis*.

A preferred embodiment of the process is that firstly mannose-6-phosphate and GTP are incubated in the presence of phosphomannomutase (*ManC*) and GDP-D-mannose synthase (*ManB*), GDP-D-mannose is optionally separated and used for the further synthesis and the desired resultant product, for example GDP-L-fucose is isolated.

Furthermore a preferred embodiment of the process according to the invention is when a buffer solution containing all starting substances or substrates is continuously percolated over a solid support material on which the (synthesizing) enzymes are immobilized.

Furthermore it has turned out to be advantageous according to the invention when the process is carried out as a batch process or continuously in an enzyme-membrane reactor.

A further subject matter of the invention is a process for coupling the GDP-6-deoxyhexoses produced according to the invention to glycosides, oligosaccharides or polysaccharides in the presence of a protein having glycosyltransferase activity. In particular the invention concerns a process for L-fucosylation or D-perosaminylation by glycosyl transfer onto suitable substrates and by providing adequate amounts of GDP-activated hexose such as GDP-L-fucose. The glycosyl transfer preferably occurs enzymatically i.e. by means of proteins which have fucosyltransferase and/or perosaminetransferase activity.

GDP-L-fucose can for example be used for the enzymatic synthesis of oligosaccharides e.g. of 2-fucosyl-beta galactosides or 3-fucosyl-beta-N-acetylgalactosamines using suitable fucosyltransferases; GDP-D-perosamine can likewise be used for the glycosylation of suitable receptor molecules such as oligosaccharides or secondary metabolites (e.g. macrolides such as perimycin) by means of suitable glycosyltransferases e.g. perosaminyltransferase from *Vibrio cholerae* O1 or other gram-negative or gram-positive bacteria.

Hence in addition to a process for the recombinant production of enzymes for the biosynthesis and transfer (glycosyltransferases) of GDP hexoses, the present invention also concerns a process for the enzymatic production of guanosine diphosphate-D-mannose, guanosine diphosphate-6-deoxy-D-hexuloses (e.g. GDP-4-keto-6-deoxy-D-mannose), guanosine diphosphate-6-deoxy-L-hexoses (e.g. GDP-L-fucose) and guanosine diphosphate-4,6-dideoxy-D-hexosamines (e.g. GDP-D-perosamine). A particular advantage of the invention is that GDP-activated sugars that could previously not be produced

in large amounts can now be prepared on a preparative scale and that in addition the building blocks obtained can be used further for the in vitro or in vivo synthesis of valuable active substances. The process is characterized by the isolation of suitable genes from bacteria and their incorporation by genetic engineering into new host organisms preferably under the control of suitable control elements (e.g. promoters) for a stronger and more controllable expression of the gene products or into new metabolic relationships with other genes in order to be utilizable for the said purposes and uses.

Legends for the figures

- Figure 1: Enzymatic synthesis of GDP- β -L-fucose and GDP-D-perosamine
Figure 2: recombinant plasmid pCAW20.1
Figure 3: recombinant plasmid pCAW19.1
Figure 4: recombinant plasmid pCAW21.1
Figure 5: recombinant plasmid pCAW22.1
Figure 6: recombinant plasmid pCAW13.1
Figure 7: recombinant plasmid pCAW14.1
Figure 8: recombinant plasmid pCAW21.2
Figure 9: recombinant plasmid pCAW22.2

The process of the present invention is described in the following in more detail on the basis of examples.

Example 1

Culture of *E. coli* strains, preparation of the plasmid DNA and isolation of DNA fragments

E. coli DH5 α and *E. coli* BL21(DE3) were preferably incubated at 37°C in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride). Plasmid-carrying bacteria were kept under the selection pressure of antibiotics (100 μ l/ml ampicillin; 30 μ l/ml chloramphenicol). The culture was carried out on a circular shaker at 270 rpm. Preparations which had been incubated for at least 12 h were referred to as an overnight culture.

Cells from a 1.5 ml overnight culture incubated under selection pressure were used to prepare plasmid DNA. The plasmids were isolated by the method of alkaline SDS-lysis (Birnboim, H.C., Doly, J., Nucleic Acid Res. 7, 1513, 1979).

Restriction endonucleases used exclusively according to the manufacturer's instructions (Gibco BRL, Eggenstein) to hydrolyse vector DNA. 5 U (units) of the respective restriction endonuclease was used for the restriction of 10 μ g plasmid DNA and incubated for 2 h at 37°C. In order to ensure complete hydrolysis the same amount of restriction endonuclease was added a second time and incubated again for at least 1 h.

The cleaved DNA was separated electrophoretically with the aid of a 1 % horizontal agarose gel. The gel pieces which contained the DNA fragments were cut out with a sterile scalpel for elution. The DNA fragments were

eluted from the agarose according to the instructions of the JETsorb kit (Genomed, Bad Oeynhausen).

Example 2

Isolation of chromosomal DNA (modified, Pospiech, A., Neumann, B., TIG 11, 217-218, 1995)

For *E. coli* DH5 α cells a 1.5 ml overnight culture was grown at 37°C in LB medium and harvested by centrifugation (5 min, 7000 rpm). The cell sediment was resuspended in 567 μ l TE buffer and incubated for 1 h at 37°C together with 30 μ l SDS (10 %), 20 μ l lysozyme solution (20 mg/ml) and 3 μ l proteinase K (20 mg/ml). 100 μ l 5 M sodium chloride solution and 80 μ l CTAB solution (hexadecyltrimethylammonium bromide) were subsequently added and inverted several times and incubated at 65°C for 10 min. After addition of 800 μ l chloroform/isoamyl alcohol, it was centrifuged for 5 min (3000 rpm) and the aqueous phase was mixed with the same volume of phenyl/chloroform in a new vessel. The phases were separated again by centrifugation and the aqueous phase was added to 0.6 parts by volume 2-propanol and the precipitated DNA was centrifuged. The DNA was washed once with 1 ml ethanol (70 %) and after drying at RT it was resuspended in 100 μ l TE buffer (10 mM Tris/HCl pH 8, 1 mM EDTA).

Example 3

Polymerase chain reaction

PCR was used for the specific in vitro amplification of

selected DNA regions

Vent DNA polymerase was used for the reactions according to the manufacturer's instructions (New England Biolabs, Schwalbach). The reaction was carried out in a thermocycler (Biometra, Göttingen).

Amplification of a DNA section by means of the PCR technique (Polymerase Chain Reaction, Saiki et al., 1985) was carried out in a 100 μ l standard mixture (Tab.1).

Table 1

Composition of the PCR mixtures

	gmd	wcaG	manB	manC	rfbD	rfbE
polymerase buffer (10x)	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l
dNTP mix (1.2 mM)	16 μ l	16 μ l	16 μ l	16 μ l	16 μ l	16 μ l
primer* (gene start)	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
primer* (gene end)	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
DMSO	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l	10 μ l
magnesium sulfate (100 mM)	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
vent polymerase	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
chromosomal DNA	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l	1 μ l
water	59 μ l	59 μ l	59 μ l	59 μ l	59 μ l	59 μ l

*The primers were diluted 1:10 before being used in the PCR. In the PCR mixture the concentration corresponded to 50 pmol/100 μ l, OD260 = 1.0/100 μ l = 100 pmol. The total volume of the PCR mixture was in each case 100 μ l and was overlayed with 60 μ l sterile mineral oil.

The PCR mixtures for the amplification of particular DNA regions were subjected to the temperature programmes (Tab. 2) shown in the table.

Table 2

Summary of the PCR programme data

	gmd		wcaG		manB		manC		rfbE		rfbD	
	time (sec)	T* (°C)	time (sec)	T* (°C)	time (sec)	T* (°C)	time (sec)	T* (°C)	time (sec)	T* (°C)	time (sec)	T* (°C)
1st Step: Hot start	300	98	300	98	300	98	300	98	300	98	300	98
2nd Step** Denaturation	60	95	60	95	60	95	60	95	60	95	60	95
3rd Step: Annealing	45	50	45	45	50	57	50	62	45	48	45	45
4th Step: DNA synthesis	60	72	60	72	70	72	70	72	60	72	60	72
5th Step: Denaturation	60	95	60	95	60	95	60	95	60	95	60	95
6th Step: Annealing	45	60	45	56	50	61	50*	71	45	57	45	56
7th Step: DNA synthesis	60	72	60	72	70	72	70	72	60	72	60	72
8th Step:	pause	12	pause	12	pause	12	pause	12	pause	12	pause	12

* The temperature change (ramping rate) between the individual steps was 2°C/s.

** Polymerase was added after a hot start during the first 95°C step.

The PCR programme cycle was selected such that steps 2 - 4 were repeated 6 times in one run and steps 5 - 7 were repeated 30 times.

Table 3: PCR primers used

Name	Gene/remarks	Sequence*
PMANB1	<i>manB</i> , <i>NdeI</i> cleavage site	5'GCCTGAAAAAGGGTAAC <u>CATATG</u> AAAAA3'
PMANB2	<i>manB</i> , <i>BamHI</i> cleavage site	5'GGGGTAAGGGAGGATCCGACATTAC3'
PMANC1	<i>manC</i> , <i>NcoI</i> cleavage site	5'TTCGGGGATAACCATGGCGCAGTCG3'
PMANC2	<i>manC</i> , <i>BamHI</i> cleavage site	5'ACACCGCGGATCCGCATTCAATGCCC3'
CAMD1	<i>gmd</i> , <i>NdeI</i> cleavage site	5'ACAGAGGAATAACATATGTCAAAAGTCGC3'
CAMD2	<i>gmd</i> , <i>Bg/II</i> cleavage site	5'CCAGCAATAAAAGATCTTTGTTTACTCATGC3'
CAMD3	<i>wcaG</i> , <i>NdeI</i> cleavage site	5'ATCGCGCTGGAGTCATACATATGAGTAAAC3'
CAMD4	<i>wcaG</i> , <i>Bg/II</i> cleavage site	5'ACGTAAAAAGATCTTTACCCCGAAA3'
CAVC1	<i>rfbD</i> , <i>NdeI</i> cleavage site	5'GGATATTTACATATGAATAAAAAAGTTG3'
CAVC2	<i>rfbD</i> , <i>Bg/II</i> cleavage site	5'CAGGAATCATTTAAAGATCTCACTCTAC3'
CAVC3	<i>rfbE</i> , <i>NdeI</i> cleavage site	5'GTGAGGTCCTTCATATGATTCTGTAT3'
CAVC4	<i>rfbE</i> , <i>BamHI</i> cleavage site	5'GGAGGTAAGGATCCCAACTACTA3'

*Newly formed cleavage sites are shown in the table and are underlined in the nucleotide sequence.

Example 4

Cloning the reading frame of *manB*, *manC*, *gmd*, *rfbD*, *rfbE* and *wcaG* in expression vectors

The reading frames isolated by PCR were cloned into expression vectors for the overexpression of ManB, ManC, Gmd, RfbD, RfbE and WcaG in *E. coli*. In this case the expression vectors pET11a and pET16b from the Novagen Company (Studier, F.W. et al., Methods Enzymol. 189, 113-130, 1990) were preferably selected for this. These vectors are strong cloning and expression systems for recombinant proteins in *E. coli*.

For cloning into the vectors these were linearized with the aid of hydrolysis by the restriction endonucleases *NdeI* or *NcoI* and *BamHI*. The fragments of the PCR products of *manB*, *manC*, *gmd* and *wcaG* hydrolysed with *NdeI* or *NcoI* and *Bg/II* or *BamHI* were linearized in the expression vectors that had been previously hydrolysed with *NdeI* or *NcoI* and *BamHI* (pCAW13.1 (*rfdD*); pCAW14.1 (*rfdE*); pCAW19.1 (*manB*); pCAW20.1 (*manC*); pCAW21.1 (*gmd*); pCAW22.1 (*wcaG*); pCAW21.2 (*his-gmd*); pCAW22.2 (*his-wcaG*), see Fig. 2-9) and transformed in *E. coli*. Cloning into the *NdeI* or *NcoI* cleavage site of the pET vectors ensured that the start codon is retained and is present on the vector at an optimal distance to the Shine-Dalgarno sequence.

The leader sequence (*his*) of the vector pET16b enables fusion of 12 histidine residues to the N-terminus of the overexpressed protein which was a prerequisite for purification of the protein by means of an Ni-agarose column. Recombinant proteins formed in this manner are referred to in the following as His-Gmd or His-WcaG and the genes coding for these proteins are referred to as *his-gmd* or *his-wcaG*.

Ligation: The fragments and vectors to be ligated were purified by elution (example 1) from the agarose gels. In the case of ligations of DNA fragments with overhanging ends (sticky ends) the fragment to be ligated was used in a four-fold excess relative to the cleaved vector and incubated for 4 h at RT with 1 U T4-DNA ligase.

Transformation using *E. coli* cells: The competent cells (Hanahan, D., J. Mol. Biol. 166, 557-580, 1983) were

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thawed on ice and 2-20 μ l DNA solution was added. After an incubation period of at least 30 min on ice, the cells were heated for 90 s to 42°C (heat shock) and subsequently placed for at least 2 min on ice. For the regeneration 800 μ l SOC-medium (2.0 % tryptone, 10 mM NaCl, 2.5 mM KCl, 0.5 % yeast extract, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM D-glucose) was added by pipette to the cells and incubated for 45 min at 37°C. 100-1000 μ l of this cell suspension was plated on selection agar plates and stored overnight at 37°C.

Example 5

DNA sequencing

The DNA sequencing of the isolated reading frame was carried out with recombinant pETIIa plasmids according to the method of Sanger et al., (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467, 1977). For the automated sequence analysis with the A.L.F. express sequencer (Pharmacia, Freiburg), the sequence reaction was carried out with fluorescein-labelled "termi" and "promo" primers and the thermo sequenase fluorescent labelled primer cycle sequencing kit using 7-deaza-dGTP according to the manufacturer's instructions (Amersham, Braunschweig).

termi primer 5'GCTAGTTATTGCTCAGCGGTG3'

promo primer 5'GAAATAAATACGACTCACTATAGGG3'

Example 6

Overexpression of ManB, ManC, Gmd, RfbD, RfbE, WcaG, His-Gmd and His-WcaG

The gene products can be preferably overexpressed in

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E. coli BL21 (DE3) with the aid of a T7-RNA polymerase/promoter system (Studier et al., 1990). For this purpose the corresponding gene was cloned behind the $\phi 10$ promoter into the MCS of the vectors pET16b or pET11a (see example 4) which have a suitable SD sequence at an optimal distance from the start codon of the target gene. The resulting recombinant plasmids were incorporated by transformation into competent cells of the *E. coli* strain BL21 (DE3).

Overexpression in *E. coli* BL21 (DE3) pLysS: LB medium (containing ampicillin, chloramphenicol) was inoculated to an OD_{540nm} of 0.05 with an overnight culture of the strain containing the appropriate plasmid and incubated in a shaker at 37°C up to an OD_{540nm} of 0.6 - 0.8. The T7-RNA polymerase was induced by adding 1.0 mM isopropylthiogalactoside (IPTG). The cells were harvested 90 min after addition of IPTG.

In order to obtain protein extracts from *E. coli* overexpression clones the cells were harvested by centrifugation and washed twice with cell lysis buffer. 1.5 ml cell lysis buffer was added to 1.0 g cells for the resuspension. Two alternative methods were used to lyse the cells and the choice depended on how much buffer was necessary for the resuspension. With a volume of less than 5 ml the cells were lysed with the aid of ultrasound in which case the cells were sonicated for 5 min with ultrasound (50 cycles, 15 s pulses and 15 s interval) and simultaneously cooled with an ice/water mixture. The extract was examined under a microscope to monitor the completeness of the lysis.

If the cells had resuspended in a volume of more than 5 ml lysis buffer, they could be lysed twice at 1300 psi by a French press (American Instrument Company, Maryland, USA).

The suspensions containing the lysed cells were centrifuged in an SS-34 rotor (Sorvall, DuPont, Bad Nauheim) for 30 - 45 min at 16000 rpm which sedimented cell fragments. The enzyme proteins were prepared in a highly purified form by classical methods of enzyme preparation or alternatively as His Tag fusion proteins (see example 7) and preserved in a stable form.

Table 4

Data for the genes and the expected gene products

Gene or gene product	size of the PCR product	size of the gene	calculated protein size	protein size in the SDS-PAGE
<i>manB</i> /ManB	1412 bp	1371 bp	50430 Da	= 50 kDa
<i>manC</i> /ManC	1486 bp	1437 bp	52996 Da	= 53 kDa
<i>gmd</i> /GmD	1025 bp	1122 bp	42020 Da	= 42 kDa
<i>gmd</i> /His-Gmd			44620 Da	= 45 kDa
<i>rfbD</i> /RfbD	1154 bp	1122 bp	42026 Da	= 42 kDa
<i>rfbE</i> /RfbE	1277 bp	1104 bp	40984 Da	= 40 kDa
<i>wcaG</i> /WcaG	1154 bp	966 bp	36090 Da	= 35 kDa
<i>wcaG</i> /His-WcaG			38690 Da	= 37 kDa

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Example 7

Purification of recombinant proteins

The purification of recombinant proteins is preferably facilitated by a C- or N-terminal fusion with histidine-containing oligopeptides (example 4). The purification was carried out with the aid of the Ni-NTA agarose from the Qiagen Company (Haan) and in accordance with the QIAexpress protocol. This affinity chromatography is based on the binding of the nickel ions of the Ni-NTA agarose to the His-tag of the specially constructed recombinant protein (His-Gmd, His-WcaG). An FPLC system was used for the purification which was composed of a liquid chromatography controller (LCC-500 plus, Pharmacia Company), two piston pumps (P500, Pharmacia Company), a flow-through UV monitor (UV-1,1280nm, Pharmacia Company), a 2-channel recorder (Rec 482, Pharmacia Company) and a fraction collector (Frac 100, Pharmacia Company).

Example 8

Gel electrophoretic isolation of proteins

Denaturing separation of proteins in SDS polyacrylamide gels and their staining with Coomassie dye

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Table 5

Composition of an acrylamide/bisacrylamide protein gel

	Separating gel (12.5 %)	Collecting gel (6 %)
water	6.6 ml	5.3 ml
collecting gel buffer (1.25 M Tris/HCl pH 8.8)	-	2.5 ml
separating gel buffer (1.5 M Tris/HCl pH 6.8)	5.0 ml	-
10 % (w/v) SDS	210 μ l	105 μ l
acrylamide-bisacrylamide sol. (29:1)	8.0 ml	2 ml
ammonium persulfate (50 mg/ml)	210 μ l	100 μ l
tetramethylethylene diamine	8 μ l	8 μ l

Electrophoresis was carried out using the SERVA Blue-Vertical 100/C apparatus (BioRad, Munich) (gel form, 80 x 100 x 0.75 mm). The protein concentration of the samples to be analysed was determined by a protein assay (BioRad, Munich) in which BSA was used to prepare a calibration line. The VIIIL Dalton Marker (14.2 kDa - 66 kDa) from Sigma (Deisenhofen) was used as a standard for the molecular weights of the separated proteins.

Example 9

Determination of enzyme activities

Determination of phosphomannomutase activity

The phosphomannomutase activity was determined according to Verseck, S. et al., (Glycobiology 6, 591-597, 1996).

Enzyme mixture:

Tris/HCl, pH 8.0	50 mM
MgCl ₂	10 mM
NADP ⁺	1 mM
glucose-1,6-biphosphate	0.25 mM
mannose-1-phosphate	1 mM
crude extract, ManC	variable
glucose-6-phosphate-dehydrogenase	0.5 U/ml
phosphomannose isomerase	0.5 U/ml
phosphoglucose isomerase	0.5 U/ml
final volume	500 μ l

The reaction was started by adding mannose-1-phosphate. The course of the reaction was monitored spectroscopically at 30°C by means of the absorbance at $\lambda_{340\text{nm}}$.

Determination of GDP-D-mannosepyrophosphorylase activity.

The GDP-D-mannosepyrophosphorylase activity was determined using the PP_i reagent from Sigma (Deisenhofen).

Enzyme mixture:

PP _i reagent	170 μ l
Tris/HCl, pH 8.0	50 mM
MgCl ₂	10 mM
GTP	2 mM
mannose-1-phosphate	10 mM
crude extract ManB	variable
final volume	500 μ l

The reaction was started by addition of mannose-1-phosphate. The decrease of NADH at 37°C was monitored spectroscopically at $\lambda_{340\text{nm}}$. The molar extinction coefficient for NADH₂ was $\epsilon_{340} = 6.22 \times 10^6 \text{ l}/(\text{mol} \times \text{cm})$.

Determination of the GDP-D-mannose-4,6-dehydratase activity

The GDP-D-mannose-4,6-dehydratase activity was determined according to (Kornfeld et al., 1965).

Enzyme mixture:

Tris/HCl, pH 7.5	50 mM
GDP-D-mannose	4 mM
crude extract Gmd or RfbD	variable
final volume	300 ml

50 μl sample was removed at defined times, added to 950 μl 1 N NaOH and incubated for a further 20 min at 37°C. The absorbance was measured at $\lambda_{320\text{nm}}$ ($\epsilon_{320} = 4600 \text{ l}/(\text{mol} \times \text{cm})$). In the negative control GDP-D-mannose was replaced by water.

Determination of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase activity

Enzyme mixture:

Tris/HCl, pH 7.5	50 mM
GDP-4-keto-6-deoxymannose	2 mM
NADPH ₂	1 mM
crude extract, WcaG	variable
final volume	0.5 ml

The reactions were started by adding the crude extract to be tested. The decrease of NADPH_2 was measured at $\lambda_{340\text{nm}}$ ($\epsilon_{340} = 6.22 \times 10^6 \text{ l/mol} \times \text{cm}$) at a temperature of 37°C .

Determination of the GDP-D-perosamine synthase activity

Enzyme mixture:

Tris/HCl, pH 7.5	50 mM
GDP-4-keto-6-deoxymannose	2 mM
L-glutamate	2.5 mM
pyridoxal phosphate	8 mM
magnesium chloride	1 mM
crude extract. RfbE	variable
final volume	0.5 ml

The amino acid L-glutamate was used as an amino donor.

The enzyme mixture was incubated at 37°C and the reaction was stopped by heating for 1 min to 100°C in a water bath. After removing the protein by centrifugation, the solution was subjected to a HPLC analysis.

The respective overexpression clones are named RfbD, RfbE, ManB, ManC, WcaG and Gmd (see example 6).

Example 10

Mixture for the preparative conversion of D-mannose-6-phosphate into GDP-D-mannose

Mixture for the preparative conversion using ManB and ManC

Tris/HCl, pH 8.0	50 mM
mannose-6-phosphate	150 μ mol
MgCl ₂	10 mM
crude extract ManB	120 nkat
crude extract ManC	120 nkat
GTP	150 μ mol
final volume	6 ml

A crude extract of *E. coli* BL21 (DE3) pLyss pCAW19.1 or pCAW20.1 was used for the conversion into GDP-D-mannose. The mixture was incubated for 1 h at 37°C. The reaction was terminated by boiling for 1 min. The proteins were separated overnight by ultrafiltration in microsep tubes (exclusion size 3 kDa; Amicon, Heidelberg) at 8000 rpm (SS-34 rotor; Sorvall DuPont, Bad Nauheim).

Example 11

Preparative conversion of GDP-D-mannose into GDP-4-keto-6-deoxymannose

Mixture for the preparative conversion using Gmd or RfbD

Tris/HCl, pH 7.5	300 μ mol
GDP-D-mannose	165 μ mol (100 mg)
crude extract Gmd or RfbD	120 nkat
final volume	6 ml

A crude extract of *E. coli* BL21 (DE3)pLySS pCAW21.1 or 2 or pCAW13.1 was used for the conversion of GDP-D-mannose. The mixture was incubated for 1 h at 37°C. The reaction was terminated by boiling for 1 min. The proteins were separated overnight by ultrafiltration in microsep tubes (exclusion size 3 kDa; Amicon, Heidelberg) at 8000 rpm (SS-34 rotor; Sorvall DuPont, Bad Nauheim). The supernatant was used further for the preparative synthesis of other GDP hexoses such as GDP-L-fucose or GDP-D-perosamine.

Example 12

Preparative conversion of GDP-4-keto-6-deoxymannose into GDP-L-fucose

Mixture for the preparative conversion using WcaG:

Tris/HCl, pH 7.5	400 μ mol
GDP-4-keto-6-deoxymannose	82 μ mol (50 mg)
NADPH ₂	123 μ mol
crude extract WcaG	3.5 U
final volume	8 ml

In order to regenerate oxidized nicotinamide adenine dinucleotide phosphate (NADP⁺) or nicotinamide adenine dinucleotide (NAD⁺) it was reduced for example by enzymatic reaction with isocitrate catalysed by isocitrate dehydrogenase.

The GDP-4-keto-6-deoxymannose was derived from a preparation described in example 11. Crude extracts of *E. coli* BL21 (DE3)/pLySS pCAW22.1 (WcaG) were used for

the conversions. These mixtures were incubated for 1 h at 37°C. The reaction mixture was then cooled on ice and 150 μ l 0.3 M perchloric acid was added. The proteins precipitated in this manner were removed by centrifugation at 30000g for 1 h. The supernatant was then neutralized with 1 M potassium hydroxide.

Example 13

Preparative conversion of GDP-4-keto-6-deoxymannose into GDP-D-perosamine

Mixture for the preparative conversion using RfbE:

Tris/HCl, pH 7.5	400 μ mol
GDP-4-keto-6-deoxymannose	82 μ mol (50 mg)
L-glutamate	123 μ mol
crude extract RfbE	3.5 U
pyridoxal phosphate	100 μ mol
final volume	8 ml

The GDP-4-keto-6-deoxymannose was derived from the preparation described in example 11. Crude extracts of *E. coli* BL21 (DE3)/pLysS pCAW14.1 (RfbE) were used for the conversions. These mixtures were incubated for 1 h at 37°C. The reaction mixture was then cooled on ice and 150 μ l 0.3 M perchloric acid was added. The proteins precipitated in this manner were removed by centrifugation at 30000g for 1 h. The supernatant was then neutralized with 1 M potassium hydroxide.

Example 14

In addition to the enzyme reactions described in examples 10-13 in a batch process, the reaction was also carried out with immobilized enzymes around which the substrate and buffer solution flowed preferably at a temperature of 30-37°C. The enzymes are for example immobilized by binding the His-tag fusion proteins (HisGmd, His-WcaG) to a Ni-NTA agarose column (Qiagen, Hilden) or by using a membrane reactor.

Example 15

Purification of GDP-hexoses

DOWEX ion exchange chromatography

In order to separate the by-product NADP^+ or NAD^+ and the residual substrate NADPH_2 or NADH_2 in the synthesis of GDP-L-fucose or pyridoxal phosphate, L-glutamate and α -ketogutarate in the synthesis of GDP-D-perosamine, an anion exchange chromatography was selected using a 1 x 8 DOWEX resin (mesh = 200-400; Serva, Heidelberg) with formate as the counterion. The resin was filled into a SR 25/50 column (Pharmacia, Freiburg). The gel bed height was 12.5 cm and the total volume was 61 ml. An FPLC system (Pharmacia, Freiburg) with a UV monitor filter for $\lambda_{254\text{nm}}$ was used for the separation. The elution was carried out as follows:

Elution volume (ml):	Dowex buffer A (%):
1200	50
1400	100 l

The total volume was 1400 ml and the flow rate was 6 ml/min. 5 ml fractions were collected. The elution was carried out at 5°C. This was followed by a HPLC analysis of the fractions after which the fractions which contained GDP-activated hexose were pooled. Subsequently this solution was concentrated to 10-20 ml in a high vacuum (rotary-gate valve vacuum pump RD4, Vacuubrand GmbH+Co, Wertheim) while stirring at ca. 25°C. After each run the column was regenerated with 185 ml 4 M formic acid and rinsed with H₂O until a neutral pH was measured.

Gel filtration

In order to desalt the fraction obtained by ion-exchange chromatography, a sephadex G-10 column (SR 25/100 column, Pharmacia, Freiburg) was used. The gel bed height was 81 cm and the total volume was 398 ml. The GDP-activated hexose was detected by means of a UV monitor (Uvicord SII, I_{254nm}, Pharmacia, Freiburg) and a 2-channel recorder (Rec 482, Pharmacia, Freiburg). The samples were collected with a fraction collector (Frac 100, Pharmacia, Freiburg). The concentrated fraction of the anion-exchange chromatography was applied to the column with a peristaltic pump (pump P-1; Pharmacia, Freiburg) and at a flow rate of 0.5 ml/min. The elution was carried out with H₂O firstly at a flow rate of 0.5 ml/min for 20-30 ml, afterwards at 1 ml/min. This was followed by a HPLC analysis of the fractions after which the fractions containing GDP-activated hexose were pooled.

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Resalting with an anion exchange membrane

In order to exchange the remaining ammonium formate by NaCl, the gel filtration fraction was pumped at 10 ml/min onto the membrane anion exchange module Q15 (Sartorius, Göttingen) (pump P-1; Pharmacia, Freiburg). Afterwards the anion-exchange module was rinsed with 20-50 ml H₂O and subsequently the activated hexose was eluted from the membrane with 150 mM NaCl. The flow rate was 10 ml/min. Afterwards this solution was concentrated to 10-20 ml/min in a high vacuum (rotary-gate valve vacuum pump RD4, Vacuubrand GmbH+Co, Wertheim) while stirring at ca. 25°C. After each run the membrane was regenerated with 20-30 ml 0.2 M NaOH and rinsed with H₂O until a neutral pH was measured.

Gel filtration and freeze drying

The solution obtained after resalting was desalted with a sephadex G-10 column as described above.

After desalting the pool of GDP-activated hexose was frozen in liquid nitrogen and freeze-dried at RT (Cryograph LCD-1, Christ GmbH, Osterode am Harz and rotary-gate valve vacuum pump RD4, Vacuubrand GmbH+Co, Wertheim).

Example 16

High pressure liquid chromatography (HPLC)

High pressure liquid chromatography was used to monitor the reaction and to analyse the nucleotide-activated

sugars. The HPLC separations were carried out using an instrument from the Beckmann Company (Beckmann Instruments, Munich) composed of a UV detector 166, pump module 125 and auto sampler 502. The following separation system was used (Payne, S.M. and Ames, B.N., Anal. Biochem. 123, 151-161, 1982):

reversed phase chromatography:

column: Eurospher 100 C18; grain size 5 mm; 250 x 4.6 mm
(Knauer, Berlin)

mobile solvent A: potassium phosphate buffer, pH 6 30 mM
 tetrabutylammonium hydrogen sulfate 5 mM
 acetonitrile 2 %

mobile solvent B: acetonitrile 100 %

elution programme:

flow rate: 1.5 ml/min
from 0 % to 40 % mobile solvent B: 60 min
100 % mobile solvent A: 15 min

All mobile solvents were filtered and degassed before use in a sterile filter unit containing an MF12 membrane (diameter 0.2 μ m; Schleicher & Schüll, Dassel). In all chromatographies the sample loop had a volume of 20 μ l. Detection was at 260 nm. The elution profiles were evaluated using the computer programme Gold Version 7.11U (Beckmann Instruments, Munich). The nucleotide-activated sugars were identified by co-chromatography and by comparing the retention times with standard substances. A three point calibration of the peak areas

versus the concentration was carried out using standard substances in order to quantify the sugars.

Table 6

Retention times of the UV-active substances

Sample	Retention times
GDP-D-mannose	15.1 min
GDP-D-4-keto-6-deoxymannose	21.6 min
GDP-beta-L-fucose	19.2 min
GDP-D-perosamine	5.5 min
GDP	22.7 min
GMP	9.7 min
NADP ⁺	25.6 min
NADPH ₂	39.6 min
pyridoxal phosphate	47.9 min

Example 17

NMR spectroscopy

The GDP-activated sugars were identified by NMR spectroscopy. The ¹H, ¹³C and ³¹P spectra were recorded on a 400 MHz instrument (Bruker AC 400, Bruker-Franzen Analytik Co., Bremen). The samples to be measured were dissolved in D₂O. The measurements were carried out at room temperature. The ¹H-NMR data for bis(triethylammonium)-β-L-fucopyranosyl-guanosine-5'-pyrophosphate from the chemical synthesis was available as a reference for GDP-β-L-fucose (Schmidt, R. et al., Liebig's Ann.

Chem., 121-124, 1991).

Example 18

In vivo production of GDP hexoses and of fucosylated or perosaminylated oligosaccharides

Cloned genes (see example 4) are cloned into vectors under the control of constitutive or inducible promoters in a common transcription unit and transformed in microorganisms preferably *E. coli*, *Streptomyces sp.* or *Saccharomyces cerevisiae*. The genes are preferably cloned under the control of promoters with a low expression rate such as e.g. *lacP* in order to ensure a constant optimal synthesis of the enzymes in a fermenter culture or in a reactor with immobilized cells. Alternatively the natural genes with their own promoters were also used. The activated sugars are transferred intracellularly with the aid of suitable glycosyl-transferases e.g. WebH (galactoside-2-L-fucosyl-transferase) from *Yersinia enterocolitica* or from *Escherichia coli* (RffT) onto suitable substrates e.g. β -galactoside which are either produced biosynthetically in the host cell or added from the outside.

Claims

1. Process for the enzymatic production of a guanosine diphosphate(GDP)-6-deoxyhexose, wherein GDP-D-mannose or a precursor thereof is incubated in the presence of one or several enzymes which have GDP-D-mannose-4,6-dehydratase (Gmd, RfbD) activity and optionally GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (WcaG) or GDP-4-keto-6-deoxy-D-mannose-4-aminotransferase (RfbE) activity and the desired product is isolated.
2. Process as claimed in claim 1, wherein GDP-4-keto-6-deoxy-D-mannose, GDP-L-fucose or GDP-D-perosamine are produced from GDP-D-mannose.
3. Process as claimed in claim 1 or 2, wherein the enzymes required for the enzymatic synthesis can be obtained by cloning the genes or DNA fragments coding for these enzymes, insertion into one or several vectors and transformation in a bacterial or fungal host cell.
4. Process as claimed in claim 3, wherein the genes or corresponding DNA regions are firstly amplified.
5. Process as claimed in claim 3 or 4, wherein the genes or DNA fragments are manB, manC, gmd, rfbD, rfbE and/or wcaG.

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6. Process as claimed in one of the claims 3 to 5, wherein the host cell is *E. coli*, *Bacillus subtilis*, *Corynebacterium* sp., *Staphylococcus carnosus*, *Streptomyces lividans*, *Saccharomyces cerevisiae*, *Schizosaccaramyces pombe*, *Hansenula polymorpha*, *Pichia stipidis*.
7. Process as claimed in claim 1, wherein mannose-6-phosphate and GTP are incubated in the presence of phosphomannomutase (ManB) and GDP-D-mannose synthase (ManC) and GDP-D-mannose or a secondary product thereof is isolated.
8. Process as claimed in one of the claims 1-7, wherein the process is carried out as a batch process.
9. Process as claimed in one of the claims 1-7, wherein the process is carried out continuously in an enzyme-membrane reactor.
10. Process as claimed in one of the claims 1-7 and 9, wherein a buffer solution containing the substrates or other starting substances is continuously percolated over a solid support material on which the enzymes are immobilized.
11. Use of a GDP-6-deoxyhexose produced as claimed in one of the claims 1 to 10 for coupling to glycosides, oligosaccharides or polysaccharides.

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12. Use as claimed in claim 11 wherein the coupling is carried out in the presence of a protein having glycosyltransferase activity.
13. Process for the production of oligosaccharides or polysaccharides containing fucose or perosamine, wherein a GDP-activated hexose is transferred onto an oligosaccharide or polysaccharide by means of a protein which has fucosyltransferase perosamine-transferase activity.

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A b s t r a c t

Process for the enzymatic preparative production of GDP-6-deoxyhexoses from GDP-D-mannose, mannose-1-phosphate or mannose-6-phosphate in the presence of suitable enzymes such as a GDP-D-mannose-4,6-dehydratase and optionally a GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase or a GDP-4-keto-6-deoxy-D-mannose-4-reductase and a process for coupling the resulting GDP-activated hexoses to oligosaccharides or polysaccharides using glycosyltransferases e.g. fucosyltransferases.

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Fig. 1

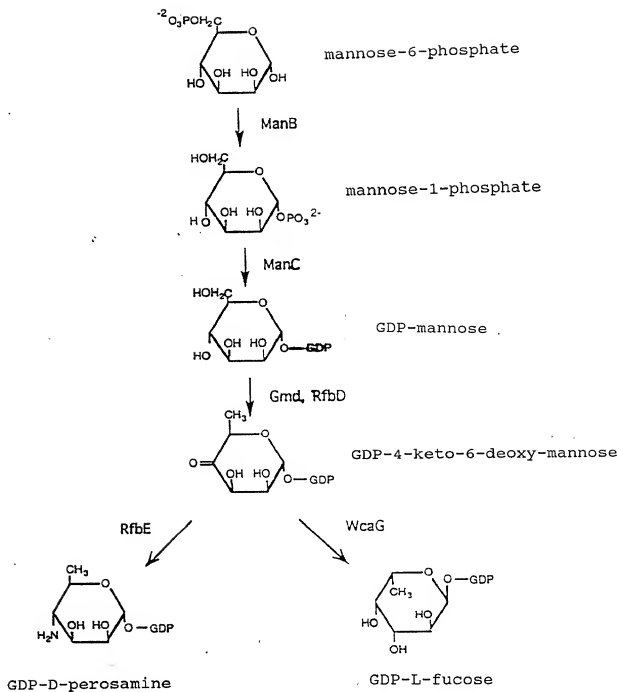


Fig. 2

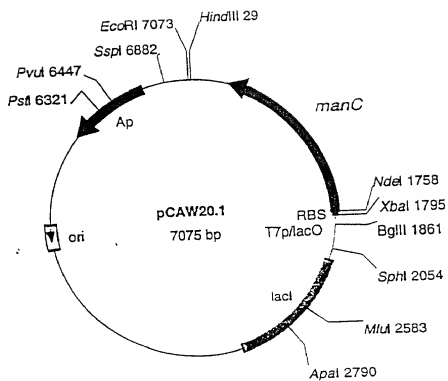


Fig. 3

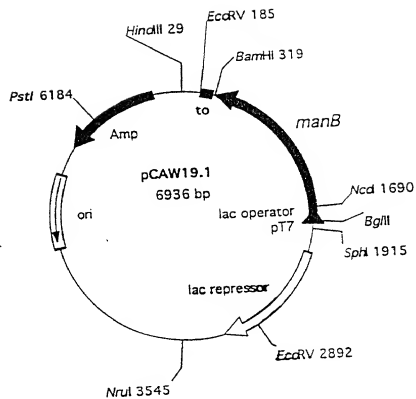


Fig. 4

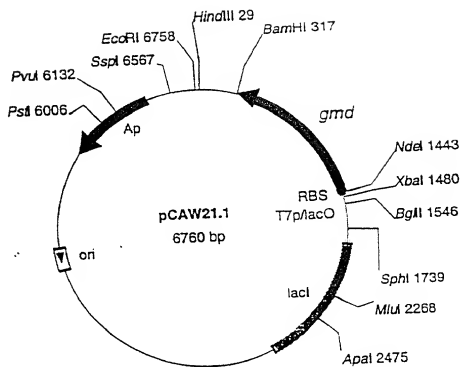


Fig. 5

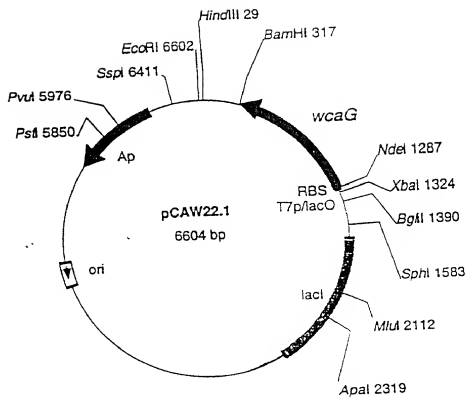


Fig. 6

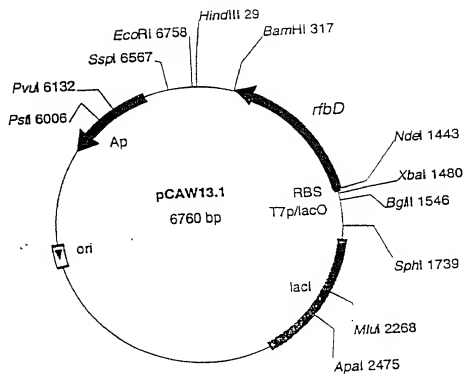


Fig. 7

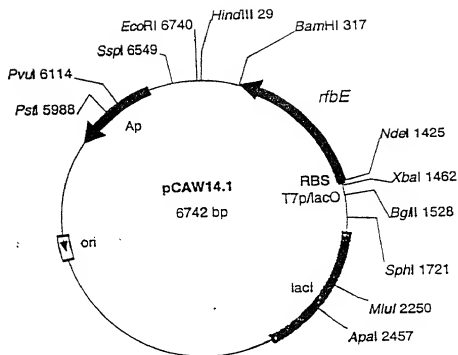


Fig. 8

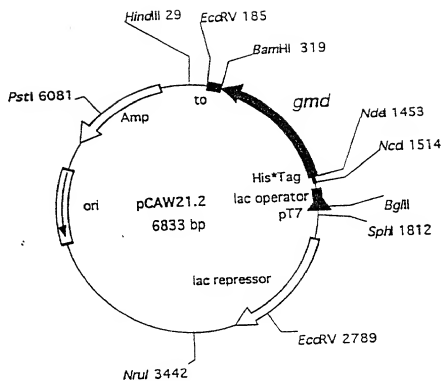
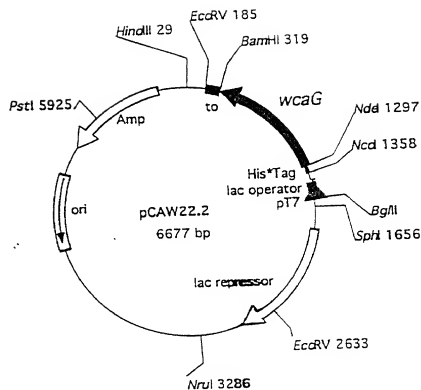


Fig. 9



Docket No.
RDID0011US

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR THE ENZYMATIC PRODUCTION OF GUANOSINE DIPHOSPHATE-6-DEOXYHEXOSES AND THEIR USE FOR THE PRODUCTION OF OLIGOSACCHARIDES

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on February 17, 2000 as United States Application No. or PCT International

Application Number 09/485,896

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

PCT/EP98/05242

PCT

18 August 1998

☐

(Number)

(Country)

(Day/Month/Year Filed)

197 35 994.9

Germany

19 August 1997

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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